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*Published in:*

Veterinary Immunology and Immunopathology

*DOI:*

[10.1016/j.vetimm.2016.10.010](https://doi.org/10.1016/j.vetimm.2016.10.010)

*Publication date:*

2016

*Citation for published version (APA):*

Jones, G. J., Steinbach, S., Sevilla, I. A., Garrido, J. M., Juste, R., & Vordermeier, H. M. (2016). Oral vaccination of cattle with heat inactivated Mycobacterium bovis does not compromise bovine TB diagnostic tests. *Veterinary Immunology and Immunopathology*, 182, 85-88. <https://doi.org/10.1016/j.vetimm.2016.10.010>

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## Short communication

Oral vaccination of cattle with heat inactivated *Mycobacterium bovis* does not compromise bovine TB diagnostic testsGareth J. Jones<sup>a,\*</sup>, Sabine Steinbach<sup>a,1</sup>, Iker A. Sevilla<sup>b</sup>, Joseba M. Garrido<sup>b</sup>, Ramon Juste<sup>b</sup>, H. Martin Vordermeier<sup>a</sup><sup>a</sup> TB Immunology and Vaccinology, Department of Bacteriology, Animal and Plant Health Agency, New Haw, Addlestone, Surrey KT15 3NB, United Kingdom<sup>b</sup> Depto. Sanidad Animal – Animal Health Dept., NEIKER-Tecnalia, 48160 Derio, Bizkaia, Spain

## ARTICLE INFO

## Article history:

Received 23 August 2016

Received in revised form 5 October 2016

Accepted 19 October 2016

## Keywords:

Bovine TB

Diagnostic tests

Heat killed *M. bovis*

Vaccination

## ABSTRACT

In this study we investigated whether oral uptake of a heat inactivated *M. bovis* wildlife vaccine by domestic cattle induced systemic immune responses that compromised the use of tuberculin or defined antigens in diagnostic tests for bovine TB. Positive skin test and blood-based IFN- $\gamma$  release assay (IGRA) results were observed in all calves vaccinated via the parenteral route (i.e. intramuscular). In contrast, no positive responses to tuberculin or defined antigens were observed in either the skin test or IGRA test when performed in calves vaccinated via the oral route. In conclusion, our results suggest that the heat inactivated *M. bovis* vaccine could be used to vaccinate wildlife in a baited form in conjunction with the following in cattle: (i) continuation of existing tuberculin skin testing or novel skin test formats based on defined antigens; and (ii) the use of IGRA tests utilizing tuberculin or defined antigens.

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## 1. Introduction

Several wildlife reservoirs have been implicated in the maintenance of cattle TB, including the Brushtail possum (*Trichosurus vulpecula*) in New Zealand (Pfeiffer et al., 1995), white-tailed deer (*Odocoileus virginianus*) in the USA (Schmitt et al., 1997), African buffalo (*Syncerus caffer*) in South Africa (De Vos et al., 2001), Eurasian badgers (*Meles meles*) in the Republic of Ireland and United Kingdom (Cheeseman et al., 1989), and Eurasian wild boar (*Sus scrofa*) in Spain (Vicente et al., 2007). As such, vaccination of wildlife species to reduce *M. bovis* infection prevalence has been proposed as a tool to support TB control programmes. Oral delivery of Baccille Calmette–Guerin (BCG), a live avirulent strain of *M. bovis*, resulted in reduced incidence and/or severity of tuberculous lesions in wild boar (Ballesteros et al., 2009), ferrets (Qureshi et al., 1999), Brush-tail possums (Tompkins et al., 2013), Eurasian badgers (Corner et al., 2010) and White-tailed deer (Nol et al., 2008) following experimental challenge with *M. bovis*. Importantly, this protection was also evident when BCG was delivered in ‘bait’ form (Ballesteros et al., 2009; Garrido et al., 2011; Nol et al., 2008). However, it has been

proposed that using inactivated vaccines would have an advantage for TB control in wildlife, in that they would be environmentally safer and more stable under field conditions compared to live BCG (Garrido et al., 2011). One such vaccine, based on heat inactivated *M. bovis* strain 1403 delivered in bait form, provided protection from *M. bovis* challenge in wild boar (Garrido et al., 2011). The concern, however, is that bystander consumption of these baits by cattle may sensitise these animals to diagnostic surveillance tests for bovine TB. To test this hypothesis, we investigated whether oral uptake of the heat inactivated *M. bovis* wildlife vaccine by domestic cattle induced systemic immune responses that compromised the use of either tuberculin or defined antigens in diagnostic assays for bovine TB, e.g. skin test or blood-based IFN- $\gamma$  release assays (IGRA).

## 2. Material and methods

## 2.1. Cattle

Calves (aged 5–7 months) were obtained from TB-free herds located in non-endemic areas of the UK. All animals were housed at the Animal and Plant Health Agency for the duration of the study, and procedures were conducted within the limits of a United Kingdom Home office license under the Animal (Scientific Procedures) Act 1986, which were approved by the APHA Animal Welfare and Ethical Review Body (AWERB) committee.

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## 2.2. Vaccination

Six calves were immunised with  $10^6$ – $10^7$  CFU of the heat killed *M. bovis* vaccine by either the oral or intramuscular route according to the supplier's guidelines (Neiker, Spain). The vaccine dose was chosen as it replicated that used in previous wild boar oral bait vaccine studies (Beltran-Beck et al., 2014; Garrido et al., 2011). The oral vaccine consisted of 0.2 ml of the inactivated *M. bovis* suspension and 4.8 ml of sterile PBS, which was administered in the lateral region of the mouth after slightly raising the head of the calves. For parenteral immunisation, 0.2 ml of the inactivated *M. bovis* suspension was homogenised with 0.8 ml of Montanide ISA 50 V2 (Seppic, France) and administered via the intramuscular route.

## 2.3. Skin tests

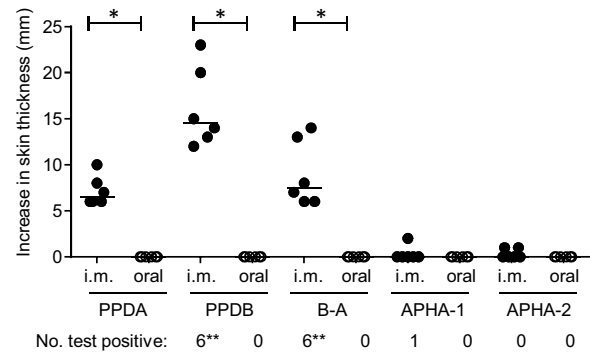
Six weeks post vaccination, all animals received intradermal injections (0.1 ml volume) in the neck with the following test reagents: (i) avian tuberculin (PPDA; 2500 IU [Prionics]); (ii) bovine tuberculin (PPDB; 3000 IU [Prionics]); (iii) APHA-1 (protein cocktail consisting of ESAT-6, CFP-10 and Rv3615c); and (iv) APHA-2 (protein cocktail consisting of ESAT-6, CFP-10, Rv3615c and Rv3020c). All recombinant proteins were obtained from Lionex (Germany) and used at a concentration of 10 µg of individual protein per injection. Skin induration was measured at the injection sites prior to and 72 h after the skin test, and the results expressed as the difference in skin thickness between the two readings ( $\Delta$  mm). The criteria for a positive skin test with the different reagents were as follows: (i) SIT responses were considered positive if  $\Delta$  skin thickness for PPDB is  $\geq 4$  mm; (ii) SICCT responses were considered positive if  $\Delta$  skin thickness for PPDB – PPDA is  $>4$  mm ('standard' OIE and UK test interpretation); and (iii) APHA-1 and APHA-2 responses were considered positive if  $\Delta$  skin thickness is  $\geq 2$  mm.

## 2.4. Whole blood IGRA

Whole blood IGRA was performed on samples collected at pre and post vaccination time points. Whole blood samples were stimulated for 24 h with bovine and avian tuberculin (300 IU/ml and 250 IU/ml respectively) or with peptide cocktails containing the following defined antigens: (i) E/C (ESAT-6 and CFP-10 [APHA]) (Vordermeier et al., 2001); (ii) Rv3615c (APHA) (Sidders et al., 2008); (iii) PC-EC (ESAT-6 and CFP-10 [Prionics]); and (iv) PC-HP (ESAT-6, CFP-10 and 4 additional mycobacterial gene products [Prionics]). All peptides were used at a concentration of 5 µg/peptide/ml. Samples were also stimulated with RPMI 1640 alone or 10 µg/ml pokeweed mitogen (Sigma-Aldrich) to act as negative and positive controls respectively. IFN- $\gamma$  levels in the plasma supernatant were quantified using the Bovigam ELISA kit (Prionics). Responses to the tuberculin reagents were considered positive if the O.D. for PPDB stimulated cultures minus the O.D. for PPDA stimulated cultures was  $>0.1$ , whilst responses to the peptide cocktails were considered positive if the O.D. for peptide stimulated cultures minus the O.D. for unstimulated cultures was  $>0.1$ .

## 2.5. Statistical analysis

Statistical analysis was performed using GraphPad Prism 6 software (GraphPad Software Inc.). Comparison of increases in skin thickness was performed using the Mann Whitney test, while the proportion of animals testing positive in skin tests or IGRA were compared using the Fisher's exact test.



**Fig. 1.** Skin test responses in cattle vaccinated with heat inactivated *M. bovis*. Six calves were vaccinated either by the intramuscular (closed circles) or the oral (open circles) route, and the skin test results are expressed as the difference in skin thickness (mm) between the pre and post skin test readings. Each circle represents an individual animal, while the horizontal line provides the median value. The number of test positive animals for the SIT (PPDB), SICCT (B-A) and defined antigen reagents (APHA-1 and APHA-2) were determined as described in the material and methods section. \*  $p < 0.01$ , Mann Whitney test, \*\*  $p < 0.01$ , Fisher's exact test (versus oral vaccination).

## 3. Results and discussion

As shown in Fig. 1, vaccination via the intramuscular route induced significantly greater ( $p < 0.01$ , Mann Whitney test) increases in skin thickness to PPDA, PPDB and B-A compared to the oral route. Furthermore, significant differences ( $p < 0.01$ , Fisher's exact test) in the proportion of animals testing positive to the tuberculin reagents were observed between the different groups, where all six calves immunised via the intramuscular route showed positive skin test reactions when using the SIT or SICCT test readouts (PPDB and B-A respectively), while no positive skin test reactions were observed in any of the calves vaccinated via the oral route when using these readouts. These results are similar to that recently reported in red deer, where oral vaccination with the heat killed *M. bovis* vaccine did not induce false-positive responses to the tuberculin skin test (Lopez et al., 2016). The defined antigen skin test reagents APHA-1 and APHA-2 have been developed as potential alternatives to tuberculin-based test reagents, and data from APHA has demonstrated that these defined antigen reagents induced positive skin test reactions in *M. bovis*-infected cattle (APHA-1 [ $n = 75$ ]: test sensitivity of 85.3% [95%CI of 75.3–92.4%]; APHA-2 [ $n = 26$ ]: test sensitivity 89.7% [95%CI of 72.7–97.8%], data not shown). In this present study, limited skin test reactions to these reagents were observed in animals immunised with heat killed *M. bovis*. With the exception of one animal immunised via the intramuscular route testing positive to APHA-1, none of the calves vaccinated with heat killed *M. bovis*, via either the oral or intramuscular route, tested positive to either of the defined antigen skin test reagents. Whole blood IGRA tests using tuberculin and defined antigen reagents were also performed pre- and post-vaccination. All 12 animals demonstrated IFN- $\gamma$  responses to the positive mitogen control (data not shown). None of the animals vaccinated by the oral route tested positive in the IGRA assay to the tuberculin reagents at any time point (Table 1). Similarly, no responses were observed to the defined antigen peptide cocktails, with the exception of one animal that tested positive to the PC-HP peptide cocktail at week 7 post-vaccination. In contrast, a significantly greater ( $p < 0.01$ , Fisher's exact test) proportion of test positive results were observed in the intramuscular vaccinated group, where all six animals showed positive IGRA test results to the tuberculin reagents 2 weeks post vaccination, which persisted for the duration of the experiment. These results are consistent with a study in wild boar where parenteral, but not oral, vaccination induced PPDB-specific IFN- $\gamma$  production (Garrido et al.,

**Table 1**  
IGRA responses in cattle vaccinated with heat inactivated *M. bovis*.

Antigen	Route <sup>a</sup>	Week 0	Week 2	Week 4	Week 6	Week 7	Week 8
B-A <sup>b</sup>	i.m.	<b>1/6</b>	<b>6/6**</b>	<b>6/6**</b>	<b>6/6**</b>	<b>6/6**</b>	<b>6/6**</b>
	oral	0/6	0/6	0/6	0/6	0/6	0/6
E/C <sup>c</sup>	i.m.	0/6	<b>3/6</b>	<b>6/6**</b>	<b>6/6**</b>	<b>6/6**</b>	<b>6/6**</b>
	oral	0/6	0/6	0/6	0/6	0/6	0/6
Rv3615c <sup>c</sup>	i.m.	0/6	0/6	0/6	0/6	<b>1/6</b>	<b>1/6</b>
	oral	0/6	0/6	0/6	0/6	0/6	0/6
PC-EC <sup>c</sup>	i.m.	0/6	<b>1/6</b>	<b>5/6*</b>	<b>4/6</b>	<b>6/6**</b>	<b>3/6</b>
	oral	0/6	0/6	0/6	0/6	0/6	0/6
PC-HP <sup>c</sup>	i.m.	0/6	<b>2/6</b>	<b>6/6**</b>	<b>5/6*</b>	<b>6/6*</b>	<b>4/6</b>
	oral	0/6	0/6	0/6	0/6	<b>1/6</b>	0/6

Bold denotes where positive responses were observed.

\* $p < 0.05$ , \*\*  $p < 0.01$ , Fisher's exact test (versus oral vaccination).

<sup>a</sup> Cattle were vaccinated via the intramuscular (i.m.) or oral route.

<sup>b</sup> Response considered positive if O.D. PPDB – O.D. PPDA  $> 0.1$

<sup>c</sup> Response considered positive if O.D. antigen – O.D. nil  $> 0.1$

2011). Frequent responses to the defined antigen peptide cocktails were also observed following intramuscular immunisation (Table 1). Responses to the E/C peptide cocktail were first seen 2 weeks post vaccination, which persisted throughout the course of the experiment in all animals. Similar results were seen with the PC-EC and PC-HP peptide cocktails; although the frequencies of response were lower at some time points when compared to the E/C cocktail. In contrast, only one animal tested positive to the Rv3615c peptide cocktail. It is interesting to note that this response first occurred at week 7 post vaccination, which was one week after the skin tests were performed. We also observed an increase in the proportion of animals in the intramuscular vaccination group testing positive to the PC-EC and PH-HP reagents at week 7 compared to week 6, although this did not achieve statistical significance (at the  $p < 0.05$  level, Fisher's exact test) and did not persist to week 8. These results are consistent with the speculation that the skin tests induced a transient boost in IGRA responses to the defined antigens, but further experiments with greater numbers of animals would be required to confirm this.

In conclusion, our results support a recommendation that the heat inactivated *M. bovis* vaccine could be used to vaccinate wildlife in a baited form in conjunction with the following in cattle: (i) continuation of existing tuberculin skin testing or novel skin test formats based on defined antigens; and (ii) the use of IGRA tests utilizing tuberculin or defined antigens. However, these recommendations come with some caveats. Firstly, we applied only a single oral dose of this vaccine to cattle. The effect of oral vaccine dose on skin test reactivity has been investigated in cattle vaccinated with BCG (Buddle et al., 2005; Wedlock et al., 2011), which showed a trend for increased proportions of animals testing positive in tuberculin skin tests following vaccination with higher doses of vaccine. Secondly, we chose to assess skin test responses at week 6 post vaccination as we deemed this sufficient time for vaccination with heat inactivated *M. bovis* to induce a systemic cell mediated immune response (as supported by the data in Table 1 and Fig. 1 for intramuscular vaccinated animals). It is possible that induction of systemic immunity following oral vaccination with heat inactivated *M. bovis* may be delayed, as previously observed in mice (Aldwell et al., 2003b), possums (Aldwell et al., 2003a) and cattle (Buddle et al., 2005) following oral BCG vaccination, although these studies employed a live vaccine (i.e. BCG) and their relevance to oral vaccination with heat killed *M. bovis* is uncertain. Thus, further studies vaccinating cattle with multiple oral doses of the heat inactivated *M. bovis* vaccine and analysing IGRA and skin test reactivity at extended time points are required to confirm the findings presented herein. Although beyond the remit of this study, we acknowledge that vaccine-challenge studies in cattle will be required to test the efficacy of this vaccine when given

by the oral route, should its application to cattle be considered. Lastly, due to its interference in diagnostic tests for bovine TB, we do not recommend using this vaccine in cattle via the parenteral route in TB control programmes combining vaccination with test and slaughter.

## Acknowledgements

We are indebted to the staff of the Animal Service Unit for their dedication to the welfare of the animals housed at APHA Weybridge. This study was funded by the EU FP7 grant WildTBvac (project ID: 613779). The funder had no involvement in the study design, data analysis, writing of the report and decision to publish.

## References

- Aldwell, F.E., Keen, D.L., Parlane, N.A., Skinner, M.A., de Lisle, G.W., Buddle, B.M., 2003a. Oral vaccination with *Mycobacterium bovis* BCG in a lipid formulation induces resistance to pulmonary tuberculosis in brushtail possums. *Vaccine* 21, 70–76.
- Aldwell, F.E., Tucker, I.G., de Lisle, G.W., Buddle, B.M., 2003b. Oral delivery of *Mycobacterium bovis* BCG in a lipid formulation induces resistance to pulmonary tuberculosis in mice. *Infect. Immun.* 71, 101–108.
- Ballesteros, C., Garrido, J.M., Vicente, J., Romero, B., Galindo, R.C., Minguijon, E., Villar, M., Martin-Hernando, M.P., Sevilla, I., Juste, R., Aranaz, A., de la Fuente, J., Gortazar, C., 2009. First data on Eurasian wild boar response to oral immunization with BCG and challenge with a *Mycobacterium bovis* field strain. *Vaccine* 27, 6662–6668.
- Beltran-Beck, B., Romero, B., Sevilla, I.A., Barasona, J.A., Garrido, J.M., Gonzalez-Barrio, D., Diez-Delgado, I., Minguijon, E., Casal, C., Vicente, J., Gortazar, C., Aranaz, A., 2014. Assessment of an oral *Mycobacterium bovis* BCG vaccine and an inactivated *M. bovis* preparation for wild boar in terms of adverse reactions, vaccine strain survival, and uptake by nontarget species. *Clin. Vaccine Immunol.* 21, 12–20.
- Buddle, B.M., Aldwell, F.E., Skinner, M.A., de Lisle, G.W., Denis, M., Vordermeier, H.M., Hewinson, R.G., Wedlock, D.N., 2005. Effect of oral vaccination of cattle with lipid-formulated BCG on immune responses and protection against bovine tuberculosis. *Vaccine* 23, 3581–3589.
- Cheeseman, C.L., Wilesmith, J.W., Stuart, F.A., 1989. Tuberculosis: the disease and its epidemiology in the badger, a review. *Epidemiol. Infect.* 103, 113–125.
- Corner, L.A., Costello, E., O'Meara, D., Lesellier, S., Aldwell, F.E., Singh, M., Hewinson, R.G., Chambers, M.A., Gormley, E., 2010. Oral vaccination of badgers (*Meles meles*) with BCG and protective immunity against endobronchial challenge with *Mycobacterium bovis*. *Vaccine* 28, 6265–6272.
- De Vos, V., Bengis, R.G., Kriek, N.P., Michel, A., Keet, D.F., Raath, J.P., Huchzermeyer, H.F., 2001. The epidemiology of tuberculosis in free-ranging African buffalo (*Syncerus caffer*) in the Kruger National Park, South Africa. *Onderstepoort J. Vet. Res.* 68, 119–130.
- Garrido, J.M., Sevilla, I.A., Beltran-Beck, B., Minguijon, E., Ballesteros, C., Galindo, R.C., Boadella, M., Lyashchenko, K.P., Romero, B., Geijo, M.V., Ruiz-Fons, F., Aranaz, A., Juste, R.A., Vicente, J., de la Fuente, J., Gortazar, C., 2011. Protection against tuberculosis in Eurasian wild boar vaccinated with heat-inactivated *Mycobacterium bovis*. *PLoS One* 6, e24905.
- Lopez, V., Gonzalez-Barrio, D., Lima-Barbero, J.F., Ortiz, J.A., Dominguez, L., Juste, R., Garrido, J.M., Sevilla, I.A., Alberdi, P., de la Fuente, J., Gortazar, C., 2016. Oral administration of heat-inactivated *Mycobacterium bovis* reduces the response of farmed red deer to avian and bovine tuberculin. *Vet. Immunol. Immunopathol.* 172, 21–25.
- Nol, P., Palmer, M.V., Waters, W.R., Aldwell, F.E., Buddle, B.M., Triantis, J.M., Linke, L.M., Phillips, G.E., Thacker, T.C., Rhyne, J.C., Dunbar, M.R., Salman, M.D., 2008. Efficacy of oral and parenteral routes of *Mycobacterium bovis* bacille Calmette-Guerin vaccination against experimental bovine tuberculosis in white-tailed deer (*Odocoileus virginianus*): a feasibility study. *J. Wildl. Dis.* 44, 247–259.
- Pfeiffer, D.U., Hickling, G.J., Morris, R.S., Patterson, K.P., Ryan, T.J., Crews, K.B., 1995. The epidemiology of *Mycobacterium bovis* infection in brushtail possums (*Trichosurus vulpecula* Kerr) in the Hauhungaroa Ranges, New Zealand. *N. Z. Vet. J.* 43, 272–280.
- Qureshi, T., Labes, R.E., Cross, M.L., Griffin, J.F., Mackintosh, C.G., 1999. Partial protection against oral challenge with *Mycobacterium bovis* in ferrets (*Mustela putorius*) following oral vaccination with BCG. *Int. J. Tuberc. Lung Dis.* 3, 1025–1033.
- Schmitt, S.M., Fitzgerald, S.D., Cooley, T.M., Bruning-Fann, C.S., Sullivan, L., Berry, D., Carlson, T., Minnis, R.B., Payeur, J.B., Sikarskie, J., 1997. Bovine tuberculosis in free-ranging white-tailed deer from Michigan. *J. Wildl. Dis.* 33, 749–758.
- Sidders, B., Pirson, C., Hogarth, P.J., Hewinson, R.G., Stoker, N.G., Vordermeier, H.M., Ewer, K., 2008. Screening of highly expressed mycobacterial genes identifies Rv3615c as a useful differential diagnostic antigen for the *Mycobacterium tuberculosis* complex. *Infect. Immun.* 76, 3932–3939.

- Tompkins, D.M., Buddle, B.M., Whitford, J., Cross, M.L., Yates, G.F., Lambeth, M.R., Nugent, G., 2013. Sustained protection against tuberculosis conferred to a wildlife host by single dose oral vaccination. *Vaccine* 31, 893–899.
- Vicente, J., Hofle, U., Garrido, J.M., Fernandez-de-Mera, I.G., Acevedo, P., Juste, R., Barral, M., Gortazar, C., 2007. Risk factors associated with the prevalence of tuberculosis-like lesions in fenced wild boar and red deer in south central Spain. *Vet. Res.* 38, 451–464.
- Vordermeier, H.M., Whelan, A., Cockle, P.J., Farrant, L., Palmer, N., Hewinson, R.G., 2001. Use of synthetic peptides derived from the antigens ESAT-6 and CFP-10 for differential diagnosis of bovine tuberculosis in cattle. *Clin. Diagn. Lab. Immunol.* 8, 571–578.
- Wedlock, D.N., Aldwell, F.E., Vordermeier, H.M., Hewinson, R.G., Buddle, B.M., 2011. Protection against bovine tuberculosis induced by oral vaccination of cattle with *Mycobacterium bovis* BCG is not enhanced by co-administration of mycobacterial protein vaccines. *Vet. Immunol. Immunopathol.* 144, 220–227.